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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
A characteristic feature of many human cancers is the inability to maintain a terminal cell cycle arrest. Indeed an increased mitotic index is used as a prognostic factor in cancer. The retinoblastoma gene is inactivated in several human cancers including those of the breast. The retinoblastoma protein (pRb) has been implicated in the maintenance of a terminal cell cycle arrest. However, in contrast to our knowledge of how pRb regulates proliferation in a cycling population, little is known about how it maintains a permanent cell cycle arrest. The proposed studies are aimed at elucidating the molecular mechanism by which pRb accomplishes this task. The significance of this line of investigation is that a determination of how pRb maintains a terminal cell cycle arrest has direct bearing on our mechanistic understanding of the role of pRb as a suppressor of tumor formation. Our working hypothesis is that pRb participates in the transcriptional repression of one or more immediate early genes required for the induction of cyclin D1. By ultimately preventing the induction of cyclin D1 this imposed a block to re-entry into the cell cycle, thus maintaining a terminal cell cycle arrest. To test this hypothesis myogenic differentiation has been used as a model. This is because it represents a differentiation system in which pRb has been implicated in a terminal cell cycle arrest both in vitro and in vivo. In the past year we have discovered that: (1) cyclin D1 is not induced following restimulation of myoblasts following culture in conditions known to induce myogenesis. This was shown to occur in a pRb- and MyoD-dependent manner. (2) Fra-1, among several immediate and delayed early genes, was the only gene not induced following restimulation of differentiated myoblasts. Our results suggest that pRb, together with MyoD, a master regulator of myogenesis prevents the induction of Fra-1 which in turn is responsible for the block to cyclin D1 expression. These results provide the basis upon which to discover the detailed mechanism by which pRb participates in a terminal cell cycle arrest.

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INTRODUCTION:

A characteristic feature of most cancers is an increase in the percentage of proliferating cells, often referred to as the mitotic index. Upon differentiation most cells in the body enter an irreversible terminal cell cycle arrest. Failure to maintain this growth arrested state is thought to contribute significantly to the development of most forms of human cancer including those of the breast. The retinoblastoma protein (pRb) has been shown to participate in the maintenance of a terminal cell cycle arrest (*1*), however, the mechanisms by it accomplishes this task are not understood. This is in contrast to the role of pRb in controlling proliferation in a cycling population, which is well characterized. The purpose of these studies are to elucidate the molecular mechanism by which pRb maintains a terminal cell cycle arrest. Initial focus is placed on the study on skeletal muscle differentiation since both *in vitro* and *in vivo* studies have clearly demonstrated a role for pRb in maintaining an arrested state following terminal differentiation of this tissue type (*1, 2*). Information gained from this analysis will then be applied to the study of mammary gland differentiation. Inactivation of the retinoblastoma gene (*Rb*) is a common event in the development of several human cancers including those of the breast. These studies thus likely have direct bearing of how loss of *Rb* contributes to the development of cancer.

BODY:

Task 1: To establish various experimental systems to study terminal growth arrest

One of our goals here was to establish the culture conditions for an irreversible growth arrest following differentiation of C2C12 myoblasts. This is a well-characterized myoblasts line that serves as a 'gold standard' for the proposed studies. This entailed growing the cells in growth medium (Dulbecco modified Eagle Medium (DMEM) plus 20% fetal bovine serum (FBS)) until they reached a state of confluence. At this time we switched the medium to differentiation medium known to induce myogenic conversion (DMEM plus 2% horse serum). Alternatively, cells were cultivated under conditions known to induce a state of quiescence, a reversible state distinct from a terminal cell cycle arrest. This was accomplished by culturing the cells under condition of low serum concentrations (DMEM plus 0.2% FBS). Following 72 hours of culture under these two conditions the cells were then restimulated with DMEM plus 20% fetal bovine serum. Upon restimulation BrdU was added to the culture medium. The ability of these cells to re-enter the cell cycle was assessed by determining whether DNA replication had occurred. The incorporation of BrdU, determined by immunofluorescence, was taken as a measure of DNA synthesis. The anticipated results was that those cells cultured in DMEM plus 2% horse serum would not re-enter the cell cycle and those cultured in DMEM plus 0.2% FBS would, which was indeed the case. However, much care and attention was devoted to optimizing the conditions for an efficient growth arrest. These included the confluence of the cells, the length of time the cells were incubated under differentiation conditions and the timing of BrdU incorporation. Culture conditions were ultimately improved to a level necessary for subsequent analyses.

In order to study both the pRb and MyoD dependence in the terminal cell cycle arrest it was necessary to perform similar experiments in fibroblasts that had been converted into myoblasts. Specifically, wild type and matched littermate *Rb*-deficient fibroblasts were employed. Initial efforts we placed on establishing the conditions for successful retrovirual infection of these cells

utilizing a virus encoding MyoD. Specifically, the appropriate titer of the virus needed to be determined as well as the level of MyoD protein obtained following infection. This was accomplished. Subsequently, optimal conditions for a terminal cell cycle arrest were established focusing on the same set of variable utilized for the C2C12 cells. Specifically, conditions were established such that *Rb*-positive fibroblasts transduced with a MyoD-encoding retrovirus cultured under conditions known to induce myogenesis would not re-enter the cell cycle following restimulation. The same cells infected with an 'empty' retrovirus re-enter S phase upon restimulation of the cells. Here entry into S phase was monitored by fluorescence-activated cell sorting (FACS). In this way the MyoD dependence for the irreversible growth arrest was established. When *Rb*-deficient fibroblasts were infected and cultured under identical conditions they did re-enter the cell cycle, thereby establishing the pRb dependence for the terminal cell cycle arrest. These experiments were successfully completed.

These research accomplishments are vital to the continuation of the project. In terms of training cell culture studies have been a cornerstone of in vitro studies for decades. The optimization of conditions to achieve a particular biological outcome reflective of what occurs in vivo provides excellent training in the 'art' of cell culture. This line of investigation also provided an excellent introduction into the use of retroviruses. Additionally, immunofluorescence technology can be used to study many cell biological processes.

Task 2: To perform analysis of immediate early and delayed early gene expression

The goal here was to test the hypothesis that following restimulation of differentiated cells one or more immediate early genes was not induced and that this correlated with the lack of induction of cyclin D1 as determined by Western blot analysis. Further, that the block to induction of immediate early gene expression occurred in a MyoD- and pRb-dependent manner. Initially, focus was placed on the characterization of C2C12 cells. Utilizing the optimized cell culture conditions it was determined that culture in DMEM plus 2% horse serum followed by restimulation in growth medium did not result in the re-induction of cyclin D1. By contrast, culture in DMEM plus 0.1% FBS followed by restimulation in DMEM plus 20% FBS did result in the induction of cyclin D1 as determined by immunofluorescent staining for cyclin D1. Immunofluorescent staining for both cyclin D1 and incorporated BrdU confirmed that the induction of cyclin D1 correlated with the ability of cells to enter S phase.

The next step was to determine whether one or more immediate early genes, which have been implicated in the transcriptional induction of cyclin D1 (*3-6*), were also not induced during restimulation of differentiated myoblasts. To this end the proteins encoded by several immediate and delayed early genes, such as c-Fos, c-Jun, Fra-1, Fra-1 and FosB, were analyzed by immunofluorescence. A considerable amount of time was devoted to trying different antibodies and developing the appropriate conditions for the detection of individual proteins. For some proteins, *e.g.* FosB, we are still trying to work out the appropriate conditions for their detection by immunofluorescence. Among the proteins analyzed only Fra-1 was not induced following restimulation (see Table 1, Figure 1 and Figure 2 in Appendix), suggesting the possibility that it was the target of pRb and MyoD in their maintenance of a terminal cell cycle arrest.

To test whether Fra-1 was indeed a target of pRb and MyoD action the fibroblast system described above was employed. *Rb*-positive fibroblasts were transduced with a MyoD or empty retrovirus and then subsequently cultured under differentiation conditions. Subsequently, the infected cells were restimulated in growth medium. The induction of cyclin D1 and Fra-1 expression was then monitored. Only in the MyoD infected and not in the empy vector infected cells was cyclin D1 and Fra-1 not induced (see Figure 3 in Appendix). This established that both cyclin D1 and Fra-1 are targets of MyoD during a terminal cell cycle arrest. To establish pRb depenence, *Rb*-deficient fibroblasts were employed. Here in both MyoD and emply vector infected cells both cyclin D1 and Fra-1 were induced following restimulation of cells previously cultured under conditions known to induce myogenesis (see Figure 3 in Appendix). Together, these results suggest that pRb and MyoD cooperate in maintaining a terminal cell cycle arrest by preventing the induction of Fra-1 and cyclin D1 following restimulation of terminally differentiated (and arrested) cells.

These research accomplishments suggest that the hypothesis being tested in this proposal is correct. Further, they establish cell culture conditions that can be utilized to determine how, mechanistically, pRb and MyoD cooperate to maintain a terminal cell cycle arrest—the goal of the next two years of research. Further, they form a working foundation on which to test the possibility that a similar mechanism is employed by other cell types, e.g. mammary epithelial cells, to maintain an arrested state—also a goal of future research.

This line of investigation provides excellent training in basic molecular biological techniques pertaining to cell cycle and differentiation—two key aspects to the study of breast cancer. It also teaches one how to carefully design an experiment to test a hypothesis.

KEY RESEARCH ACCOMPLISHMENTS:

- Cell culture conditions established to study terminal cell cycle arrest in C2C12 myoblasts
- Condition for efficient retroviral infection of *Rb*-positive and *Rb*-deficient fibroblast established
- Conditions to study terminal cell cycle arrest in retrovirally infected fibroblasts established
- Determined that the lack of induction of cyclin D1 correlates a permanent cell cycle arrest
- Determined that the block to cyclin D1 induction occurs in an pRb- and MyoD-dependent manner
- Determined that among several immediate early genes only Fra-1 is not induced following restimulation of differentiated myoblasts
- Determined that the block to Fra-1 induction, which correlates with the block to cyclin D1 induction, occurs in an pRb- and MyoD-dependent manner

REPORTABLE OUTCOMES:

- Cell lines: wild type and matched littermate *Rb*-deficient fibroblasts transducted with either a MyoD encoding retrovirus or empty vector
- Results have been presented in a public forum at the Dana-Farber Cancer Institute

CONCLUSIONS:

The inability to maintain a terminal cell cycle arrest is a cardinal feature of cancer. *Rb* is inactivated in several human cancers including those of the breast. pRb has been implicated in the maintenance of a terminal cell cycle arrest, however, the mechanism by which it accomplishes this is not known. Our studies, supported by this fellowship, suggest that pRb maintains a cell cycle arrest by blocking the expression of immediate early gene expression, which in turn is responsible for the induction of cyclin D1—an event required for re-entry into the cell cycle. The results reported in this annual summary focus on the events required for a terminal cell cycle arrest in myoblasts. However, they likely have direct bearing on cell types more prone to the development of cancer, e.g. mammary epithelial cells. Thus, the results provide a framework on which to study the process of a terminal cell cycle arrest in other cell types. Most importantly, the results reveal a novel mechanism of action for pRb in the suppression of tumor formation.

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APPENDIX:

Table 1. Induction of immediate early and delayed early genes following restimulation of C2C12 myoblasts from either a quiescent or differentiated state

Immediate or delayed early gene	Induction following resimulation from a quiescent state	Induction following restimulation from a differentiation state
Cyclin D1	+	–
c-Fos	+	+
c-Jun	+	+
Fra-1	+	–
Fra-2	+	+
JunB	+	+
JunD	+	+

C2C12 myoblasts were either differentiated in 2% horse serum or incubated in the presence of 0.1% fetal bovine serum for 72 hours. At this time very low or undetectable levels of the genes listed was observed. At this time cells were restimulated in the presence of 20% fetal bovine serum. At various times following restimulation (up to 24 hours) cells were fixed, permiablized and immunostained with antibodies to various protein encoded by the indicated immediate and delayed early genes. Following restimulation from a quiescent state all proteins were induced. By contrast, following restimulation of C2C12 cells cultured under conditions known to induce myogenesis only cyclin D1 and Fra-1 were not induced.

Figure Legends:

Figure 1. Fra-1 is not induced following restimulated of differentiation of C2C12 cells
C2C12 cells were incubated under conditions that induce myogenesis (2% horse serum; panels A-D) or that bring about a state of quiescence (0.1% fetal bovine serum; panels E-H). Differentiated or quiescent myoblasts were stimulated for four hours in the presence of 20% fetal bovine serum (panels B, D, F and H). Differentiated cells were then fixed, permiabilized and stained with an antibody to Fra-1 (rhodamine, red) or myosin heavy chain (MHC; marker of differentiation; FITC, green) (panels A and B) and counterstained with DAPI to visualize nuclei (panels C and D). C2C12 rendered quiescent with 0.1% fetal bovine serum with or without restimulation were stained with an antibody to Fra-1 (rhodamine, panels E and F) or counterstained with DAPI (panel G and H).

Figure 2. c-Fos is induced following restimulated of differentiation of C2C12 cells
C2C12 cells were incubated under conditions that induce myogenesis (2% horse serum; panels A-D) or that bring about a state of quiescence (0.1% fetal bovine serum; panels E-H). Differentiated or quiescent myoblasts were stimulated for four hours in the presence of 20% fetal bovine serum (panels B, D, F and H). Differentiated cells were then fixed, permiabilized and stained with an antibody to c-Fos (rhodamine, red) or myosin heavy chain (MHC; marker of differentiation; FITC, green) (panels A and B) and counterstained with DAPI to visualize nuclei (panels C and D). C2C12 rendered quiescent with 0.1% fetal bovine serum with or without restimulation were stained with an antibody to c-Fos (rhodamine, panels E and F) or counterstained with DAPI (panel G and H).

Figure 3. pRb and MyoD cooperate to block the induction of Fra-1 and cyclin D1 following restimulation of differentiated myoblasts
Two *Rb*+/+ and one *Rb*-/- fibroblasts lines were infected with a MyoD encoding retrovirus or empty vector control as indicated. Infected cells were then incubated under conditions known to induce myogenesis for 48 hours (2% horse serum). Cells were then either left in differentiation medium or restimulated with 10% fetal bovine serum for 8 hours as indicated. Cells were harvested and subjected to immunoprecipitation for Fra-1 or cyclin D1. Immune complexes were resolved on a denaturing gel, transferred and blotted with antibodies to Fra-1 or cyclin D1. *Rb*-positive fibroblasts shown in lanes 4 through 6 and *Rb*-deficient cells used in lanes 7 through 9 were derived from matched littermate embryos; lanes 1 through 3 employed fibroblasts from a non-littermate. Note that Fra-1 and cyclin D1 are not induced following restimulation only when both pRb and MyoD are present.

FIGURE 1

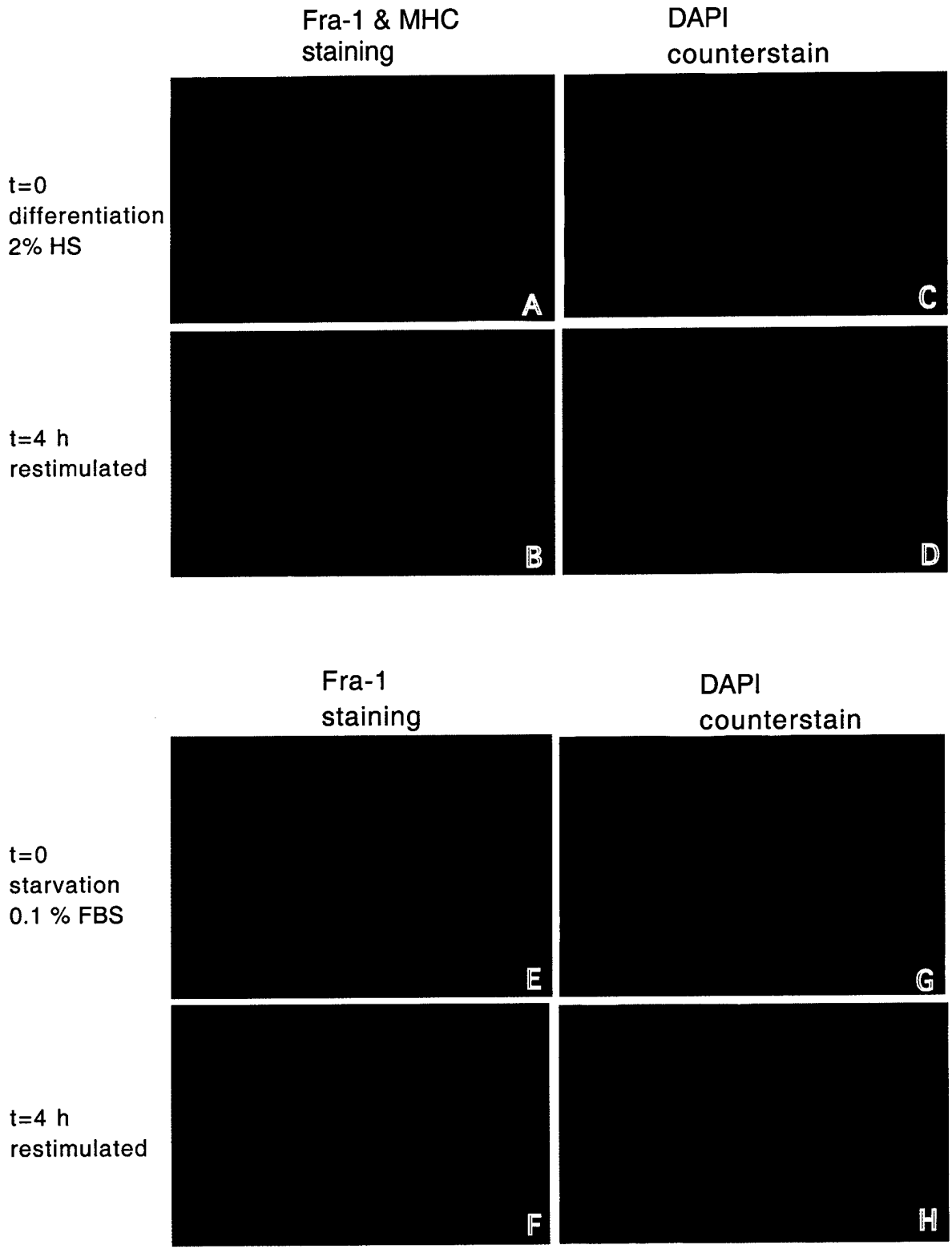
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FIGURE 2

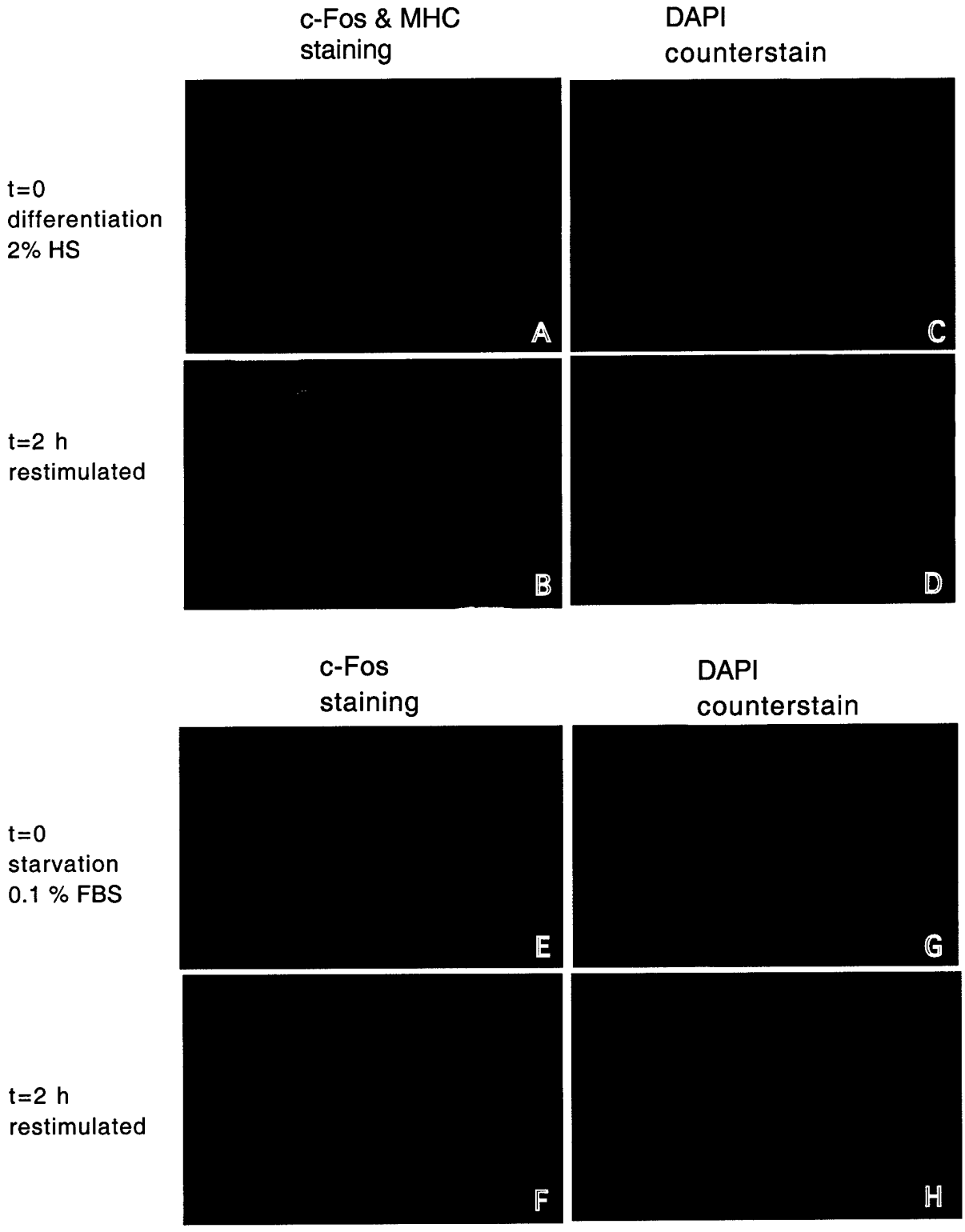
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FIGURE 3